Gynodioecy in Plantago lanceolata

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Gynodioecy in *Plantago lanceolata*. VI. Functions of cytokinins in growth, development, and reproduction of two sex types

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Theoretical models predict that male sterile plants of gynodioecious species should show at least some compensation for their disadvantage of not reproducing as males through female component of fitness, In this study, growth, development, and reproduction of a hermaphrodite and a male sterile family of *Plantago lanceolata* L. were compared under controlled conditions. The male sterile plants produced more and longer spikes and had relatively longer styles. The male sterile plants achieved their final biomass sooner, by an earlier formation of side rosettes, and flowered earlier. The hypothesis was tested as to whether cytokinins in the plants are involved as a pleiotropic factor in either or both sex expression and the various plant characteristics associated with the male sterile phenotype. The roots of the male sterile plants had higher concentrations of putative zeatin riboside than the roots of the hermaphroditic plants, as quantified by an enzyme-linked immunoassy after separation of cytokinins by high performance liquid chromatography. Spraying the plants with benzyladenine did not affect internal cytokinin concentrations or sex expression. Benzyladenine spray increased the growth rate of the main rosette and stimulated floral initiation. Our results indicate that cytokinins are possibly involved in determining the morphological differences between sex types in this species.

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Des modèles théoriques prédisent que des plantes d'espèces gynodioïques de stérilité mâle devraient au moins montrer une certaine compensation pour le désavantage de ne pas se reproduire comme mâles, par leur composante femelle d'adaptation. Dans cette étude, la croissance, le développement et la reproduction d'un hermaphrodite et d'une famille à stérilité mâle de *Plantago lanceolata* L. furent comparés sous des conditions contrôlées. Les plantes à stérilité mâle ont produit des épis plus nombreux et plus longs, et elles avaient des styles relativement plus longs. Les plants mâles stériles ont atteint plutôt leur biomasse finale suite à une formation plus hâtive des rosettes latérales, et elles ont fleuri plus tôt. L'hypothèse selon laquelle des cytokinines endogènes sont impliquées comme facteur pléiotrope dans l'expression de l'un ou des deux sexes et des diverses caractéristiques végétales associées au phénotype à stérilité mâle fut vérifiée. Les racines des plantes à stérilité mâle avaient des concentrations plus élevées en zéatine riboside que les racines des plantes hermaphrodites, tel que quantifié par un essai immunologique, après séparation des cytokinines par la chromatographie liquide à haute performance. L'arrosage des plantes avec de la benzyladénine n'a pas affecté les concentrations en cytokinines internes, ni l'expression du sexe. L'arrosage avec la benzyladénine a augmenté le taux de croissance de la rosette principale et stimulé l'initiation florale. Nos résultats indiquent que les cytokinines sont possiblement impliquées dans la détermination des différences morphologiques entre les types de sexe chez cette espèce.

[Traduit par la revue]

Introduction

In plants, two major components of fitness can be distinguished, since individual plants mostly contribute genes to the next generation through the production of both seed and pollen. The importance of distinguishing between a male and female component of fitness is particularly clear in gynodioecious species that consist of hermaphrodite and male sterile plants. Since male steriles only reproduce as females and not as males, they are at a disadvantage compared with the hermaphrodites. Theoretical models predict that without any compensation for the disadvantage of male sterility this trait would quickly disappear from the population (7, 12, 27, 32), with the amount of required compensation depending on the mode of inheritance. In *Plantago lanceolata* L. and *Thymus vulgaris* L., two wild species in which the inheritance of male sterility is well documented, a nuclearcytoplasmic pattern of inheritance is shown (14, 33). The nuclearcytoplasmic inheritance of male sterility seems to be universally present (7) and is even of fundamental importance; the primary reason for the existence of gynodioecy seems to be related to an evolutionary conflict between nuclear and cytoplasmic genes (8, 9, 12). In the case of nuclearcytoplasmic inheritance, the amount of compensation required for maintenance is smaller than the twofold difference required in the case of nuclear inheritance.

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The classical outcrossing hypothesis for gynodioecy postulates that male steriles are maintained in populations because all their progeny result from outcrossing, whereas the progeny of the hermaphrodites may partly result from self-fertilization and may then be less fit because of inbreeding depression (17, 28). However, there are two reasons why this cannot be the only mechanism involved. First, some gynodioecious species are self-incompatible (e.g., *P. lanceolata* (30)). Inbreeding depression is unlikely to be involved here, and it has been argued that the male sterility genes themselves bring compensation with them through pleiotropic effects (31). Second, from theoretical studies about the nucleocytoplasmic inheritance of male sterility (11, 12, 23), the conclusion can be drawn that for the maintenance of a joint polymorphism of nuclear and cytoplasmic variation a pleiotropic effect is required, viz., a fitness disadvantage of plants with a restorer allele in combination with a cytoplasm whose effect on male fertility is not restored by this allele (12).

In *P. lanceolata*, at least three types of male sterility are present, denoted as MS1, MS2, and MS3 (29). Each sterility type has a corresponding hermaphrodite type. Studies in the field demonstrated that the MS1 plants have more and longer spikes than H1 (hermaphrodites with the same cytoplasm as MS1) plants, and consequently form more seeds (30). The MS1 plants can also show a longer half-life time (34). These differences could contribute to a compensation for the disadvantage of male sterility. It has often been argued that pleiotropic effects are involved in the determination of the complex of traits associated with male sterility in this species (31).

The aim of the present study is to describe the differences between MS1 and H1 sex types of *P. lanceolata* in growth, development, and reproduction under controlled conditions and to search for a pleiotropic physiological factor determining these differences. Cytokinins show feminizing effects on the sex expression of plants (6, 18, 20). Therefore, the internal concentrations of the natural cytokinins zeatin and zeatin riboside were studied. Furthermore, experiments have been carried out to study the effects of application of benzyladenine (BA; a synthetic cytokinin) on growth, development, and reproduction and on the internal cytokinin concentrations.

Materials and methods

Growth conditions

Two F2 families of *P. lanceolata* were used in the present experiments. The original parents were grown from seeds collected in the Westduinen area (Goeree, The Netherlands).

The mode of inheritance of MS1 is known (29), and the two families chosen only yielded H1 and MS1 plants, respectively. Seeds were germinated in sterilized vermiculite moistened with distilled water; the seed coat of each seed was cut with a razor knife to promote germination. After 14 days, the seedlings were transferred to an aerated nutrient solution, as specified by Smakman and Hofstra (26), adjusted at pH 6.0. Four sets of 10 plants for each family were grown in 30-L tanks.

The plants were grown in a greenhouse for 64 days with a day–night temperature regime of 22:15°C. The natural photoperiod was extended to 18 h with the aid of high-pressure mercury lamps. To ensure flowering in the winter season (with a possible shortage of red light), the plants were continuously illuminated with eight 100 W incandescent lamps. The culture solution was replaced twice a week and was screened from light to prevent algal growth.

Twenty-eight days after sowing, the BA application started. Two sets of plants for each family were sprayed three times a week with a 10^{-6} M BA solution containing 0.005% (v/v) Tween-80.

Growth and development

The biomass of the leaves of each plant was estimated at 3- to 4-day intervals from 21 to 57 days after sowing. Biomasses of the main rosette and the side rosettes were estimated separately from the product of the number of leaves, the length of the longest leaf, and the width of the longest leaf. Linear regression equations were computed for the relation between the estimated weight, as described above, and actually measured fresh weights. The parameters of the regression equations were determined separately for the main rosette and the side rosettes for each combination of sex type and BA treatment on day 21, 24, 28, 31, 35, and 41 after sowing. The 24 different regression equations were all significant at least at the 0.05 level.

Other characteristics determined at 3- to 4- day intervals after sowing were the frequency of plants per sex type per BA treatment forming side rosettes, the number of side rosettes per plant, the frequency of plants flowering, the number of ears of each flowering plant, the length of the longest spike, the length of the flowering part of each spike, the length of the longest style.

Preparation of samples for determination of cytokinins

At 35, 42, and 64 days after sowing, the concentrations of zeatin (Z) and zeatin riboside (ZR) were determined in the spike, shoot, and root tissue of plants of the two sex types and from the BA treatment. The harvested plant material (10–25 g fresh weight of shoots, roots, or spikes) was homogenized in 60 mL cold ethanol and filtered. The supernatant was evaporated to dryness (30°C) and taken up in 10 mL demineralized water; 10 mL Tris-HCl was added (0.1 M, pH 8.2, containing 0.02 M $MgCl_2$). The mixture was shaken with alkaline phoshatase for 16 h at 30°C. After adjustment to pH 3.0, the mixture was extracted four times with water-saturated *n*-butanol. The collected butanol samples were evaporated to dryness, and the residual was taken up in 2 mL NH_4-formate buffer (10 mM, pH 3.7).

Purification and HPLC of plant extracts

Alkaline phosphatase treated plant extracts were purified by anion chromatography in NH_4-formate buffer (10 mM, pH 3.7) over a diethylaminoethyl cellulose column and captured with a Sep-Pak C_{18} column. Thereafter, cytokinins were washed from the cartridge with 96% alcohol (10 mL). The alcoholic solution was evaporated to dryness and stored for HPLC.

Z and ZR were separated using a μBondapak C_{18} column (Waters, 30 × 0.39 cm) eluted with 25% methanol–NH_4-formate (10 mM, pH 3.7; flowrate 1.5 mL/min). Retention times were determined using standard Z and ZR samples (purchased from Sigma). Fractions of putative Z and ZR were collected, evaporated to dryness, and taken up in 0.05% phosphate buffer saline + Tween-20 (PBST buffer, 0.01 M, pH 7.4).

Enzyme-linked immunoassay

Enzyme-linked immunoassays (ELISA) were carried out by means of Costar microtitre plates (flat bottom, 96 wells), as described by Vonk et al. (35). The following buffers were used: a coating buffer (0.05 M Na_2CO_3–$NaHCO_3$, pH 9.6), a PBST buffer, and a substrate buffer (coating buffer + 0.5 mM $MgCl_2$). For coating, each well of the microtitre plates was filled with Z or ZR conjugate (immobilized phase, 200 μL, 20 μg/mL coating buffer). To prevent evaporation, plates were stored in plastic boxes at 3°C (at least overnight) until required for use. Before use, plates were washed once with water and five times with PBST buffer (5×). Samples, in several dilutions, or standards in PBST buffer were added to the wells (100 μL). Then the required amount of antibody against Z or ZR in PBST buffer was added (100 μL). For determination of maximum antibody binding (B_o) and minimum antibody binding (B_u), PBST buffer or large excess of standard were added in separate wells. After mixing with a Titerek variable shaker (1 min), plates were then stored in covered boxes overnight at 3°C to form the immunocomplex. The plates were then washed with PBST buffer (5×), and all wells were filled with anti-rabbit IgG coupled to alkaline phosphatase (200 μL, diluted in PBST buffer 1500×). An absorbance (A) of $B_o - B_u = 1.0$ has to be found by combining the specified dilution of coupled anti-rabbit IgG

TABLE 1. F values and levels of significance of analyses of variance for the traits in Fig. 1

Source of variation	No. of days after sowing	Morphological trait[a]					
		A	B	C	D	G	H
Sex type (S)	17	21.84***	—	—	21.84***	—	—
	21	14.41***	—	—	14.41***	—	—
	24	16.38***	—	—	16.38***	—	—
	28	23.25***	—	—	26.25***	—	—
	31	19.68***	—	—	19.36***	—	—
	35	8.56**	—	—	9.39**	—	—
	38	1.36	25.54***	—	66.85***	—	—
	41	0.63	27.60***	18.96***	36.40***	—	—
	45	0.91	27.74***	21.16***	20.97***	11.67**	6.92*
	49	0.70	11.83***	8.11***	6.11*	65.68***	30.46***
	57	6.63*	4.49*	1.58***	2.41	64.88***	1.56**
Benzyladenine treatment (BA)	28	—	—	—	—	—	—
	31	0.47	—	—	0.47	—	—
	35	4.56*	—	—	2.46	—	—
	38	12.15***	1.72	—	5.48*	—	—
	41	22.32***	1.12	0.22	0.27	—	—
	45	29.27***	0.82	2.17	0.47	3.06	1.72
	49	32.68***	1.50	0.23	0.01	1.35	1.86
	57	26.10***	1.47	10.20**	0.24	0.56	0.86
S × BA interaction	28	—	—	—	—	—	—
	31	1.00	—	—	1.00	—	—
	35	0.10	—	—	0.32	—	—
	38	0.07	0.15	—	0.01	—	—
	41	0.01	0.08	2.29	1.43	—	—
	45	0.05	0.56	2.62	3.45	0.36	0.73
	49	0.01	0.02	4.97*	2.65	0.01	0.13
	57	0.12	0.41	7.59*	1.21	0.15	0.59

NOTE: For days 17, 21, 24, and 28, one-way ANOVAS were computed for the factor sex type; for the other days of measurement (after the BA application had started) two-way ANOVAS with the factors sex type and BA treatment were performed. *, significant at $p < 0.05$; **, significant at $p < 0.01$; ***, significant at $p < 0.001$; —, not enough data for computation.

[a]See Fig. 1 for list of morphological traits.

with an appropriate dilution of antibody. Plates were shaken, incubated in closed plastic boxes for 3 h at 35°C, and washed with PBST buffer (5×). Subsequently, the substrate was added to the wells (200 μL; mg/mL) and the plates were incubated for 1 h at 35°C in plastic boxes. Colour development was stopped by addition of KOH (50 μL, 5 M) to each well. Optical densities (405 nm) were measured by means of a Costar ELISA reader connected to a Hewlett Packard 85 computer for data processing. Mean standard curves were determined for Z and ZR. The percent maximum binding was calculated from the equation

$$\frac{B}{B_o} = \frac{A_B - A_{B_u}}{A_{B_o} - A_{B_u}} \times 100$$

Sigmoidal curves were linearized using the formula for logit transformation

$$\text{logit } (B/B_o) = \ln \frac{B/B_o}{100 - B/B_o}$$

No statistics were computed for the differences in cytokinin concentrations. Since one extract per group of plants was analysed in ELISA, the experimental error only reflects variation between different dilutions, instead of variation in the concentrations of the plant material.

Results

Growth and development

The MS1 plants had a significantly heavier main rosette than the H1 plants until day 35 (Fig. 1A; Table 1). The formation of side rosettes started earlier in MS1 plants (Fig. 1B; Table 1), and plants of this sex type formed significantly more and heavier side rosettes (Fig. 1C; Table 1). Therefore, the largest differences in the total vegetative shoot fresh weight occurred in the period of onset of the side rosette formation (Fig. 1D; Table 1). Flowering started earlier in the MS1 plants (Fig. 1F), and the MS1 plants produced significantly more and longer spikes (Figs. 1G, 1H; Table 1). The style length increased with the duration of flowering of each spike. After correction for the flowering stage in a two-way analysis of covariance, there was a significant difference between the sex types ($F_{[1,192]} = 386.8$; $p < 0.001$): MS1 plants had longer styles.

The application of BA significantly increased the growth rate of the main rosette from day 35 until day 57 in H1 and MS1 plants. The spraying with BA significantly decreased the fresh weight of the side rosettes at the final harvest. This reduction was more pronounced in H1 plants; the interaction between the sex type and the BA treatment was significant at day 49 and day 57 (Table 1). The application of BA accelerated flowering in both sex types (Fig. 1F), but it did not affect the number of ears and the spike length (Figs. 1G, 1H).

Cytokinin concentrations

The shoots and roots of both sex types contained more ZR than Z (Fig. 2). This difference was greater in the roots than in the shoots; the ears had about the same content of Z and ZR.

The roots of the MS1 plants had a higher ZR content than the roots of the H1 plants, and this difference increased with time.

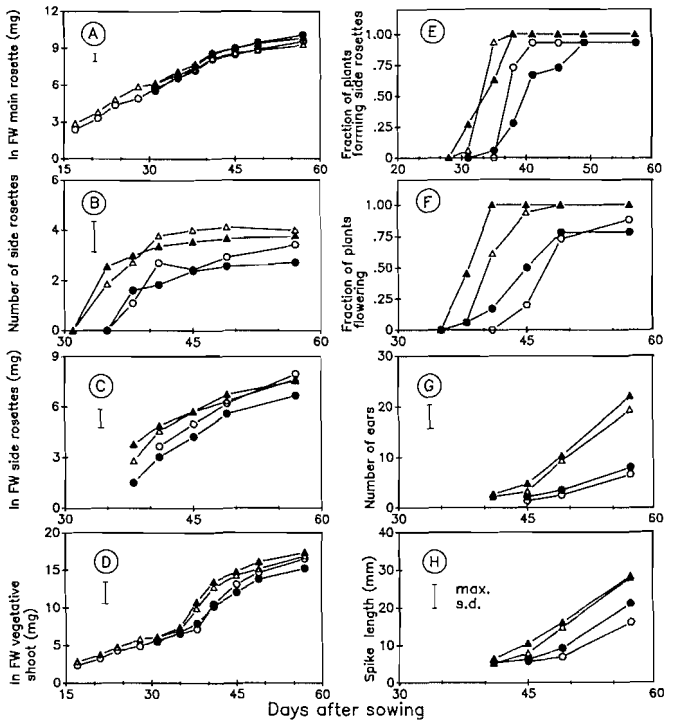

FIG. 1. Fresh weight (FW) of the main rosette (A); number of side rosettes (B); fresh weight of the side rosettes (C); fresh weight of the vegetative part of the shoot (D); fraction of plants forming side rosettes (E); fraction of the plants flowering (F); number of spikes per plant (G); and length of the longest spike at different days after sowing (H). ○, control H1 plants; △, control MS1 plants; ●, H1 plants sprayed with benzyladenine (BA); ▲, MS1 plants sprayed with BA. The error bars represent standard deviations. Tests of significance of the factors sex type and BA treatment are given in Table 1.

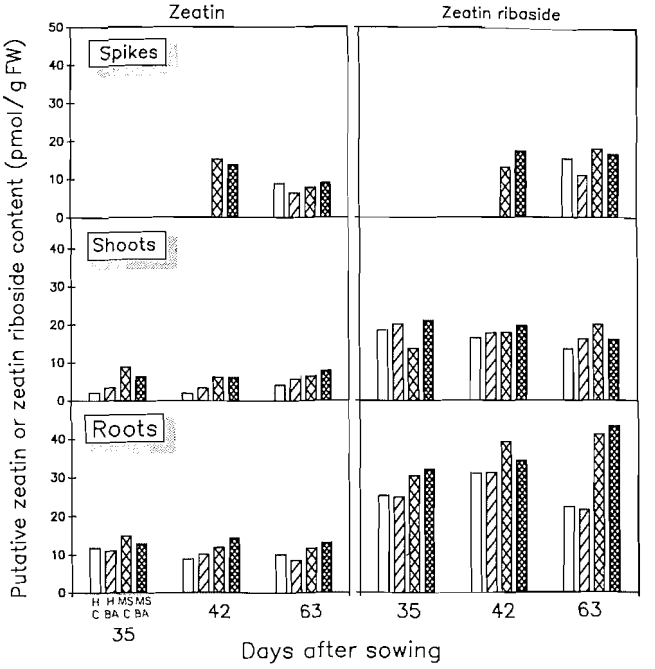

FIG. 2. The concentrations of putative zeatin and zeatin riboside in the spikes, shoots, and roots of control H1 plants (□), H1 plants sprayed with benzyladenine (BA); ▨, control MS1 plants (⊠), and MS1 plants sprayed with BA (⊠) at 35, 42, and 63 days after sowing. The concentrations were determined by enzyme-linked immunoassay (ELISA). FW, fresh weight.

The MS1 plants also had a slightly higher Z concentration in shoots and in roots. There was no clear difference between the sex types in the Z and ZR content of the ears.

Spraying the plants with BA had no consistent effect on the concentrations of Z and ZR.

Discussion

Several differences between MS1 and H1 plants were found. MS1 plants produced more and longer spikes, consistent with differences found in natural populations of this species (30). The relatively longer styles (measured at the same flowering stage) of the MS1 plants may further increase female fecundity by enhancing the fertilization chance. According to theoretical models, differences in female fecundity between male steriles and hermaphrodites as found here are required for the maintenance of gynodioecy (7, 10, 23). Similar differences in fecundity between male sterile and hermaphrodite plants have been found in other species (reviewed in ref. 21). Our present results show that the fecundity differences between MS1 and H1 plants, as found in natural populations (30, 34), are also present under controlled conditions.

The reported differences in vegetative growth characteristics could influence the survival rate of the sex types under competitive conditions. An experiment in this context was performed by Bonnemaison et al. (4) with *T. vulgaris*, where no sex type dependent survival was found in a competition experiment.

The higher ZR content in the roots of MS1 plants (Fig. 2) may be involved in determining male sterility in the MS1 plants, since cytokinins have been shown as having feminizing effects on sex expression (formation of phenocopies) in several species (6, 18, 20). However, an opposite effect of cytokinins on the sex expression of *Hordeum vulgare* was found by Ahokas (1, 2). Although our attempt to influence the internal concentrations of cytokinins and the sex expression by spraying with BA was not successful, BA addition did accelerate flowering. The determination of the sex expression may thus be regulated by other factors than cytokinins alone. Feminizing effects have also been reported for auxins and abscisic acid (5, 6, 24) and for gibberellins (3, 5, 13, 22, 25).

If the differences between the sex types are mediated by a pleiotropic effect of cytokinins, a correspondence between the effects of external application of BA and the differences between the sex types should be expected. The MS1 plants had a heavier main rosette in the first half of the experiment, and the growth of the main rosette was stimulated by BA (Fig. 1A; Table 1). Growth-stimulating effects of cytokinins have often been reported (e.g., 15, 16, 19). The earlier start of flowering in the MS1 plants corresponds with the observation that BA addition enhanced flowering (Fig. 1F). Bernier and Kinet (3) found that zeatin riboside and zeatin could promote the initiation of flowering in some long-day plants. It can be concluded that there is a concordance between the demonstrated effects of spraying with BA and the differences between the sex types in internal concentrations of cytokinins and phenotypic plant characteristics. However, further work on the physiological regulation of the developmental pathways leading to male sterile and hermaphrodite phenotypes is necessary.

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